REMARKS

Claims 1-129, 183, 232, 255 and 278 are pending. The Office Action of October 2, 2005, presents the examination of claims 67, 73-76, 255 and 278. Claims 1-66, -72, 77-129, 183 and 232 are withdrawn subsequent to restriction.

Restriction maintained

The Examiner maintains her position that the restriction requirement is proper, whereas Applicants strongly disagree, and again assert that the restriction requirement is in fact merely an election of species. Furthermore, even if the restriction is properly presented, the Examiner will have to examine additional species should the linking claims 67 and 73 be found allowable.

The Examiner asserts that the restriction groups 1-47 are not merely separate species of a generic invention because they do not meet the requirements of a Markush group. That is, the members of the claims 1-47 do not have a substantial structural feature in common or they do not have a common utility. The Examiner's position is that the "cytokine and the T-cell epitope or the different viruses do not share a common utility or a common structural feature essential to that utility." The Examiner is not correct.

The various viruses of the present invention include as a common structural feature a genome or antigenome (hereinafter merely "genome") comprising a genome of a HPIV2 virus into which has been inserted a foreign gene of another pathogen. By analogy to more traditional pharmaceutical compounds, the HPIV2 viral genome constitutes the "pharmacophore" that might be visualized as the "central" structure of the drug, *e.g.* a fused ring system, and the distinct inserted foreign genes inserted into the viral genome constitute the different "R" groups that would be appended to the ring system. The various point mutations in the viral genome might be considered analogous to insertion of different heteroatoms into the ring system.

All of these components contribute to the "common function" of presenting epitopes of the recombinant virus to the host immune sytem so as to induce an immune response to a

pathogen in the immunized host while at the same time limiting viral reproduction in the host (attenuation) so as to prevent disease caused by the viral vector itself.

Since the various viruses presently claimed do share a common structural element and a common function, consideration of the present restriction requirement as an election of species is the more proper approach to initial examination and the instant restriction requirement should be vacated.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 75 and 76 stand rejected under 35 U.S.C. § 112, first paragraph, for alleged failure of the specification to provide enabling disclosure of the full scope of the claimed invention. The Examiner is requiring deposit of the virus HPIV3 JS cp45 under the terms and conditions of the Budapest Treaty.

The required deposit has in fact been made prior to filing of this application in connection with a different, commonly owned, U.S. Patent application. A copy of the receipt for deposit of the strain is attached hereto and the required Declaration regarding availability of the deposit will be filed in a supplement to this response. Applicants submit that the filing of this Declaration will obviate this ground of rejection.

Rejection under 35 U.S.C. § 102

Claims 67, 73-75, 255 and 278 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Murphy et al. WO '078. This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

The present claim 67 recites:

> An infectious, self-replicating, recombinant human parainfluenza virus type 2 (HPIV2) comprising a PIV major nucleocapsid (N) protein, a PIV nucleocapsid phosphoprotein (P) a PIV large polymerase protein (L) and a partial or complete, polyhexameric recombinant HPIV2 genome or antigenome.

The essence of the instant rejection is that the recitation in claim 67 that the partial or complete recombinant HPIV2 genome is "polyhexameric" has been deemed to describe a property of HPIV2 that is inherent to the virus.

A property of a composition or system that is "inherent" is one that is a necessary result of the structure of the composition or circumstances of the system. It is not enough that the result is one that is possible, or even highly probable; the "inherent" result must occur. See, *e.g. Ex parte Vander Wal and Van Akkeren*, 109 USPQ 119 (BPAI 1955); *In Hansgirg v. Kemmer*, 40 USPQ 665, 667 (CCPA 1939)("Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.")

In the present instance, the Examiner should consider that while Murphy '078 shows experimental results demonstrating that replication of a minigenome using HPIV3 replication signals at the 5' and 3' ends of the virus is highest if the length of the minigenome is a multiple of six nucleotides in length, the data also show significant replication occurs at lengths that are not multiples of six. See, Fig. 5B of the reference, at data points +1 and +3. Therefore, Murphy '078 teaches that a HPIV virus having a length that is not a multiple of six nucleotides will replicate, although at a lower rate compared to a polyhexameric virus.

Furthermore, at the time the invention was made, it was not established that HPIV2 followed the "rule of six" that was deemed the norm for other HPIVs. Attached hereto as Exhibit 1 is a copy of a paper by M. Kawano et al. (*Virology* 284:99-112 (2001)). At pp. 105-106 of this paper, the authors discuss that under some circumstances, a HPIV2 virus having a genome length that is not an even multiple of six nucleotides could be isolated and could replicate efficiently in cultured cells, and state that HPIV2 "does not appear to absolutely obey to the 'rule of six,' suggesting that 6n length genome has less significance in *Rubulavirus* than in *Respirovirus*."

Thus, that HPIV2 should have a polyhexameric genome was not established as a fact at the time the present application was filed, and a polyhexameric genome or antigenome should

not be considered an inherent property of a HPIV2 virus. Accordingly, Murphy '078 should not be taken to disclose a polyhexameric genome (or antigenome) as an inherent property of HPIV2, and the instant rejection should be withdrawn.

In view of the above arguments, applicant believes the pending application is in condition for allowance.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Mark J. Nuell Reg. No. 36,623 at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37.C.F.R. §§1.16 or 1.14; particularly, extension of time fees.

Dated: March 30, 2007

Respectfully submitted,

By _____
Mark J. Nuell
Registration No.: 36,623
BIRCH, STEWART, KOLASCH & BIRCH, LLP
8110 Gatehouse Road
Suite 100 East
P.O. Box 747
Falls Church, Virginia 22040-0747
(703) 205-8000
Attorney for Applicant

Virology 284, 99–112 (2001)
doi:10.1006/viro.2001.0864, available online at http://www.idealibrary.com on IDEAL®

Recovery of Infectious Human Parainfluenza Type 2 Virus from cDNA Clones and Properties of the Defective Virus without V-Specific Cysteine-Rich Domain

Mitsuo Kawano,*[1] Masahiko Kaito,† Yuji Kozuka,* Hiroshi Komada,*‡ Naoya Noda,* Kazuyoshi Nanba,* Masato Tsurudome,* Morihiro Ito,* Machiko Nishio,* and Yasuhiko Ito*

*Department of Microbiology, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. †The Third Department of Internal Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan, and ‡Department of Microbiology, Suzuka University of Medical Science, Suzuka, Mie 510-0293, Japan

Received January 2, 2001; returned to author for revision January 26, 2001; accepted February 12, 2001

A full-length cDNA clone was constructed from the genome of the human parainfluenza type 2 virus (hPIV2). First, Vero cells were infected with recombinant vaccinia virus expressing T7 RNA polymerase, and then the plasmid encoding the antigenome sequence was transfected into Vero cells together with polymerase unit plasmids, NP, P, and L, which were under control of the T7 polymerase promoter. Subsequently, the transfected cells were cocultured with fresh Vero cells. Rescue of recombinant hPIV2 (rPIV2) from cDNA clone was demonstrated by finding the introduced genetic tag. As an application of reverse genetics, we introduced one nucleotide change (UCU to ACU) to immediate downstream of the RNA-editing site of the V gene in the full-length hPIV2 cDNA and were able to obtain infectious viruses [rPIV2V(−)] from the cDNA. The rPIV2V(−) possessed a defective V protein that did not have the unique cysteine-rich domain in its carboxyl terminus (the V-protein-specific domain). The rPIV2V(−) showed no growth in CV-1 and FL cells. Replication of the rPIV2V(−) in these cells, however, was partially recovered by adding anti-interferon (IFN)-β antibody into the culture medium, showing that the rPIV2V(−) is highly sensitive against IFN and that no growth of rPIV2V(−) in CV-1 and FL cells is mainly due to its hypersensitivity to endogenously produced IFN. These findings indicate that the V-protein-specific domain of hPIV2 is related to IFN resistance. On the other hand, the rPIV2V(−) efficiently replicated in Vero cells, which are known as a IFN-non-producers. However, the virus yields of rPIV2V(−) in Vero cells were 10- to100-fold lower than those of control rPIV2, although syntheses of the viral-specific proteins and their mRNAs in rPIV2V(−)-infected Vero cells were augmented up to 48 p.i. in comparison with those of rPIV2. Furthermore, the rPIV2V(−) virions showed anomalous in size as compared with rPIV2 virions. These results suggest that the V protein plays an important role in the hPIV2 assembly, maturation, and morphogenesis.

INTRODUCTION

Human parainfluenza type 2 virus (hPIV2) is one of the major human respiratory pathogens and a member of the genus *Rubulavirus* in the family *Paramyxoviridae*, possessing a single-stranded, nonsegmented, and negative-stranded RNA genome of ~15.6 kb. The genome of hPIV2 encodes seven mRNAs (Kawano *et al*., 1990a–c, 1991; Ohgimoto *et al*., 1990; Yuasa *et al*., 1991) and has an ~60-nt leader sequence at 3′ end and an ~20-nt noncoding trailer sequence. The gene order is 3′-(leader)-NP-V/P-M-F-HN-L-(trailer)-5′. The coding proteins are the nucleocapsid (NP), the V (V) and phospho (P), the matrix (M), the fusion (F), the hemagglutinin-neuraminidase (HN), and the polymerase protein (L). The genomic RNA of the virus [viral RNA (vRNA)] is encapsidated with the NP proteins, resulting in nuclease-resistant helical nucleocapsids, and the nucleocapsids were associated with the P and L proteins to form the ribonucleoprotein complex. The viral polymerase complex (RNA-dependent RNA polymerase), which consists of the L and P proteins, is probably thought to be responsible for RNA transcription and replication, termination, and reinitiation at gene junction, capping, and polyadenylating all mRNAs, but these have yet to be demonstrated. The vRNA also serves as a template for the synthesis of a full-length positive-sense complementary RNA, which is replicative intermediate. Most paramyxoviruses have the unique property of promoting the transcription of mRNA containing nontemplated insertions at a specific site in the P gene carrying a part of viral RNA polymerase (Thomas *et al*., 1988; Cattaneo *et al*., 1989; Vidal *et al*., 1990; Paterson and Lamb, 1990; Ohgimoto *et al*., 1990; Kondo *et al*., 1990; Kawano *et al*., 1993). In hPIV2 RNA editing, the pseudo-templated addition of two G residues produces an mRNA that encodes the protein termed P, while the unedited mRNA that is the exact copy of the P gene encodes the V protein.

The construction of functional RNA replicons from

[1] To whom reprint requests should be addressed at Department of Microbiology, Mie University School of Medicine, 2-174, Edobashi, Tsu, Mie 514-8507, Japan. Fax: +81-59-231-5008. E-mail: kawanom@doc.medic.mie-u.ac.jp

0042-6822/01 $35.00

cDNA and the successful recovery of infectious recombinant viruses have greatly facilitated the molecular genetic analyses of RNA viruses. Until recently, this technology was applicable only to positive-stranded RNA viruses because, in contrast to the situation with positive-stranded RNA viruses, the deproteinized genomic RNA of negative-stranded viruses is not infectious, and RNA, with negative or positive polarity, transcribed from full-length cDNA clones of the genomes of negative-stranded RNA viruses, is not by itself competent to initiate infection.

An efficient system completely devoid of infectious helper virus was first used for vesicular stomatitis virus (VSV), a member of the *Rhabdoviridae* family. Intracellularly transcribed synthetic RNA corresponding to the genome of a nonexpressing natural VSV copyback-type defective interfering (DI) particle was rescued by VSV proteins expressed from the plasmids (Pattnaik *et al*., 1992). This system employed bacteriophage T7 DNA-dependent RNA polymerase, which was expressed cytoplasmically from a vaccinia virus (VV) recombinant [vTF7-3 (Fuerst *et al*., 1986)]. The T7 polymerase synthesized DI particle genomic RNA from circular transcription plasmids that were introduced into the cells by transfection. Finally, in the presence of the complete complement of five VSV proteins expressed from the plasmids, the replicated DI RNA transcripts were incorporated into infectious DI particles that budded and were released from the cells.

Attempts to manipulate the full-length genome of the mononegavirus were facilitated by studies using the methods described above. These procedures were then extended to allow the rescue from the cloned DNA of infectious virus. The initial success was the recovery of rabies virus by Schnell *et al*. (1994). Their methods using a plasmid encoding a full-length antigenomic viral RNA became a key to recovery of nonsegmented and negative-stranded RNA virus from cDNA. Application of this system led to success of the rescue from the cDNA of infectious VSV, measles virus (MV), human respiratory syncytial virus (RSV), Sendai virus (SV), human parainfluenza virus type 3 (PIV3), simian virus 5 (SV5), Newcastle disease virus (NDV), and mumps virus (MuV) (Lawson *et al*., 1995; Whelan *et al*., 1995; Radecke *et al*., 1995; Collins *et al*., 1995; Garcin *et al*., 1995; Kato *et al*., 1996; Hoffman and Banerjee, 1997; Durbin *et al*., 1997; He *et al*., 1997; Peeters *et al*., 1999; Clarke *et al*., 2000).

As the applications of reverse genetics to these recombinant viruses, the elimination of alternative gene products encoded in the P genes to elucidate their roles in viral replication and pathogenesis was carried out. Recombinant viruses deleting the VSV C/C′ proteins (Kretzschmar *et al*., 1996), Sendai virus V protein (Kato *et al*., 1997), Sendai virus C/C′ proteins and C/C′/Y1/Y2 proteins (Garcin *et al*., 1997; Kurotani *et al*., 1998), and MV C protein (Radecke *et al*., 1996) have been generated. Among these deleting mutant viruses, some mutations in the Sendai virus showed significant effects on viral replication. Compared with the wild-type Sendai virus in various cell line *in vitro*, the V protein knockout [V(−)] virus was found to be either potentiated or comparable but never attenuated. However, the V(−) virus showed remarkably attenuated *in vivo* replication capacity and pathogenicity for mice (Kato *et al*., 1997). A point mutation in the Sendai virus C proteins attenuated virulence for mice but not virus growth in cell culture (Garcin *et al*., 1997). The 4C knockout [4C(−)] Sendai virus, which expresses none of the four C proteins, was further attenuated in tissue culture as compared with the C/C′(−) or V(−) virus (Kurotani *et al*., 1998).

Furthermore, among the defective C(−) viruses, only the 4C(−) virus eliminated the viral ability to prevent the interferon (IFN)-α/β-mediated responses (Gotoh *et al*., 1999). Thus, in the Sendai virus, the anti-IFN function is attributed to the C proteins; Didcock *et al*. (1999), however, have reported that the V protein of simian virus 5 belonging to the Rubulavirus inhibits IFN signaling by targeting STAT1, which is degraded by the enhanced-proteasome activity.

In this report, we described the construction and generation of hPIV2 full-length infectious cDNA clones. For the purpose of studying functions of V protein in the Rubulavirus by using this reverse genetics system, we generated a V protein-negative virus by introducing one nucleotide change to downstream of the RNA-editing site and analyzed its properties.

RESULTS

Construction of cDNA to hPIV2 genome

To construct a cDNA clone encoding the full-length hPIV2 genome, we attempted to ligate the long RT–PCR fragments produced by restriction enzymes, which cut one site on cDNA for hPIV2-vRNA. We were able to find those sites, *Sac*I on V/P gene (2673), *Kpn*I on HN gene (8137), and *Bst*PI on L gene (10,027) (Fig. 1). We synthesized appropriate oligonucleotide primer pairs as shown in Table 1 and performed the RT–PCR using vRNA as a template. Five overlapping cDNA fragments (Fig. 1) synthesized by RT–PCR were digested by unique restriction enzyme in both termini and ligated to similarly digested pUC118RVG plasmid vector (see Materials and Methods), respectively. Then, a plasmid including the full-length hPIV2 cDNA genome (pPIV2) was constructed by step-wise assembly from these subcloned plasmid DNAs. Finally, as shown in Fig. 1, we constructed pPIV2 including the hPIV2 full-length cDNA with additional genetic tag (*Not*I) flanked on its left end by a T7 RNA polymerase promoter and at the right end by a hepatitis delta virus ribozyme followed by a T7 terminator, and 3G residues were also inserted before the hPIV2 leader sequence for the purpose of increasing the transcription

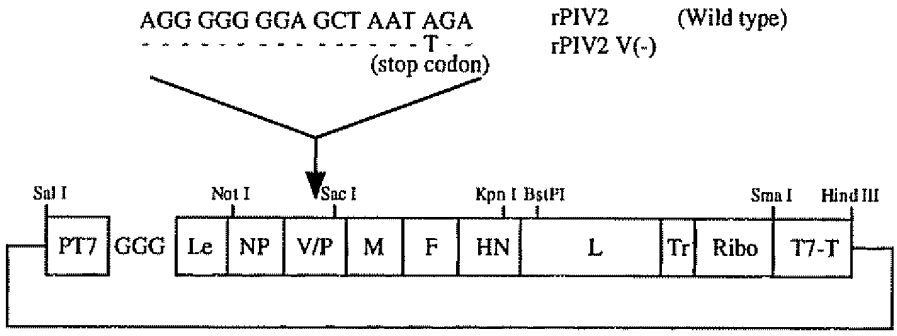

FIG 1 Schematic diagram of the full-length infectious clone, pPIV2 and pPIV2V(−), essential to making an antisense genome RNA. The full-length cDNA was constructed from five overlapping RT–PCR cDNA fragments. frg 1 (*SalI–NotI*), frg 2 (*NotI–SacI*), frg 3 (*SacI–KpnI*), frg 4 (*KpnI–BstPI*), and frg 5 (*BstPI–SmaI*) pPIV2 contains the full-length cDNA of hPIV2 genome and is flanked at one end by a T7 promoter (PT7) and at the other end by HDV ribozyme (Ribo) and a T7 terminator (T7-T) pPIV2 contains three extra G residues between PT7 and the hPIV2 leader (Le) sequence The *NotI* site added on NP gene shows the genetic tag found in the recovered viruses The arrow shows one nucleotide change introduced into pPIV2 for construction of the plasmids. pPIV2V(−), encoding defective V protein without V specific cysteine-rich C terminus

efficiency by T7 RNA polymerase Transcription of pPIV2 by T7 RNA polymerase produced the hPIV2 full-length antigenome (+) sense RNA transcript

Construction of a hPIV2 genome cDNA-retaining defective V gene

To elucidate the function of the hPIV2 V protein, we introduced a TGA stop codon in the V frame, immediate downstream of the RNA editing site by mutating ^{2507}A to T (U to A in the genomic sense) in the plasmid pPIV2, generating a full-length copy of hPIV2 antigenome (Fig 1) This mutated plasmid was designated pPIV2V(−) This mutation did not show any effect on the P frame

Recovery of infectious hPIV2 from cDNA

To recover infectious recombinant hPIV2 from cDNA, Vero cells were infected with a recombinant vaccinia virus (MVA) that expresses T7 RNA polymerase and causes lower cytopathic effects than vTF7-3 (Wyatt *et al*, 1995), and after 2 h, the cells were transfected with pPIV2 together with the plasmids that encode hPIV2 polymerase units, NP, P, and L proteins The amount of each plasmid used for hPIV2 rescue (see Materials and Methods) was referred to the success in rescuing infectious SV5 (He *et al*, 1997) The recovery procedure was as follows; Vero cells cultivated in six-well plates were transfected with the above plasmids After 6 h, the me-

TABLE 1

Primers Used to Construct the Full-Length cDNA of the PIV2 Genome

Primer 1	5′-ACGCGTCGACTAATACGACTCACTATAGGGACCAAGGGGAGAATCAGATGG-3′
	SalI PT7 Le (21 nt)
Primer 2	5′-ATAGTTTAGCGGCCGCAGATAGAGAATGATCAGATAGTAT-3′
	NotI
Primer 3	5′-ATAAGAATGCGGCCGCATCTCTATTAAGGATATTTCTAGTCTAAAG-3′
	NotI
Primer 4	5′-CAAGTCCCTTTAAGAGCTCAATGATCTCCTTCA-3′
	SacI
Primer 5	5′-TGAAGGAGATCATTGAGCTCTTAAAGGGACTTG-3′
	SacI
Primer 6	5′-ACTTGATAGGACGGTACCCATTGAGCCTCAATG-3′
	KpnI
Primer 7	5′-CATTGAGGCTCAATGGGTACCGTCCTATCAAGT-3′
	KpnI
Primer 8	5′-TTTTCTTTCTACGGTAACCATTAATTAAAATTGT-3′
	BstPI
Primer 9	5′-ACAATTTTAATTAATGGTTACCGTAGAAAGAAAA-3′
	BstPI
	SmaI
Primer 10	5′-TCCCCCGGGCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTTCGGATGCCCAGGTCGGACCGCGAGGA
	GGTGGAGATGCCATGCCGACCCACCAAGGGGAAAATCAATATGTTTTTC-3′
	Ribo (84 nt) Tr (27 nt)

dium was changed followed by incubation for a further 40 h The cells were harvested and cocultured with fresh Vero cells After 48 h-incubation, the media were harvested and then inoculated into Vero cells, which were further incubated for 48 h The supernatant was filtered through 0 22-μM filter to remove vaccinia virus and was used to further infect Vero cells After incubation for 24 h, large numbers of typical foci of hPIV2-induced syncytia with hemadsorption activity were observed (data not shown) The virus rescue from pPIV2V(-) cDNA was performed according to almost the same methods described above, but the cocultures of transfected cells with fresh Vero cells were done twice every 48 h before harvest of the media

Identification of virus rescue by RT–PCR and Western blot assay

To investigate whether the infectious viruses were produced from the cDNA, we purified reverse-transcribed vRNA from putative rPIV2- or rPIV2V(−)-infected cells using primers that annealed close to the genetic tag (*Not*I site) introduced at nt 126 to 137 The RT products were then amplified by PCR using a second primer to generate a fragment covering the genetic tag As shown in Fig 2A, an appropriately sized DNA fragment (385 bp) was found only in complete reaction When RT was omitted from the reaction, no proper-size PCR product was found (data not shown) Restriction endonuclease digestion by *Not*I showed that the expected *Not*I site was present in the RT–PCR fragment derived from vRNA of either a putative rPIV2 or rPIV2V(−) but not in that from parental hPIV2 Furthermore, the nucleotide sequencing of the RT–PCR fragments proved the existence of the additional *Not*I site in the rPIV2 and rPIV2V(−) (Fig 2B), indicating the rescue of infectious recombinant hPIV2 from the cDNA clone Then, the nucleotide sequencing of mutated site in rPIV2V(−) was performed using RT–PCR fragment covering the RNA-editing site in the P gene, and the rescue of rPIV2V(−) from the cDNA clone was also reconfirmed (Fig 2B)

To study whether the mutation (stop codon) introduced into the V gene of pPIV2 could fulfilled its function in virus transcription by the rPIV2 polymerase, we carried out the Western blot assay using the rPIV2V(−)- or rPIV2-infected Vero cell lysate and the specific monoclonal antibody against the NP or the P/V protein of hPIV2 (Tsurudome *et al*., 1989; Nishio *et al*, 1999) As shown in Fig 2C, the rPIV2V(−) was found to synthesize no detectable V protein, whereas the NP and P proteins were clearly detected in the virus-infected cells All these proteins were detectable in the rPIV2-infected cells Furthermore, the lower-molecular-weight polypeptide (MW of ~ 24K) was detected only in rPIV2V(−)-infected cells (Fig 2C), and the polypeptides were considered to be I-like protein observed in mumps virus-infected cells (Paterson and Lamb, 1990), indicating that gene manipulation of vRNA, namely, introduction of mutation, gets along well

Gene expression and replication of rPIV2 and rPIV2V(−) in Vero cells

Subsequently, we analyzed the growth of rPIV2 and rPIV2V(−) in Vero cells under multiple-cycle growth conditions (an m o i of 0 001) Figure 3A shows rPIV2V(−) exhibits a slower kinetics than rPIV2 throughout the experimental period The virus titer of rPIV2V(−) was reduced by 10- and 100-fold as many as that of rPIV2 at 48 and 72 p i , respectively, that is, the virus yield of rPIV2 was $\sim 3 \times 10^5$ $TCID_{50}$/ml at 72 h p i , while that of rPIV2V(−) was $\sim 3 \times 10^3$ $TCID_{50}$/ml at 72 h p i (Fig 3A), indicating that rPIV2V(−) is remarkably attenuated in Vero cells

Then, the viral polypeptides synthesized in rPIV2- and rPIV2V(−)-infected Vero cells under the multiple-cycle growth conditions were analyzed by Western blot assay using anti-hPIV2 NP and P/V antibodies The level of NP and P protein synthesis was much higher in the rPIV2V(−)-infected cells ≤48 p i than that in the rPIV2-infected cells (Fig 3B) These findings exhibit a striking contrast to the virus yields (Fig 3A) Interestingly, however, the protein synthesis of rPIV2 rapidly increased at 72 h p i , while the synthesis rate of virus-specific proteins was gradually increasing in rPIV2V(−)-infected cells after 48 h p i The data also confirmed that the V protein was lacking in rPIV2V(−)

In the next experiment, the level of virus transcription and genome replication in the same infected cells was analyzed by RT–PCR/Southern The kinetics patterns between the virus proteins and mRNAs syntheses paralleled On the other hand, vRNA synthesis was not augmented in rPIV2V(−)-infected cells from 24 to 48 h p i (Fig 3C) Intriguingly, though the virus yield of rPIV2V(−) at 72 h p i was as many as that of rPIV2 at 48 h p i , the synthetic quantities of the viral proteins and mRNAs of rPIV2V(−) at 72 h p i were more than those of rPIV2 at 48 h p i (Fig 3C), suggesting that the function of assembly and/or maturation of rPIV2V(−) is insufficient

Electron microscopical observation of the rPIV2 and rPIV2V(−)

To further clarify an influence of V protein loss on the viral morphology, the virions of rPIV2, rPIV2V(−), and wtPIV2 grown in Vero cells were examined by an immunoelectron microscopy using mouse anti-hPIV2 HN monoclonal antibody, followed by labeling with goat anti-mouse IgG antibody-colloidal gold particles and negative staining The wtPIV2 and rPIV2 preparations displayed a relatively homogenous population of spherical particles with a diameter of ~100 ~ 200 nm (Figs 4A, 4B, and 4D), and mean diameters of wtPIV2

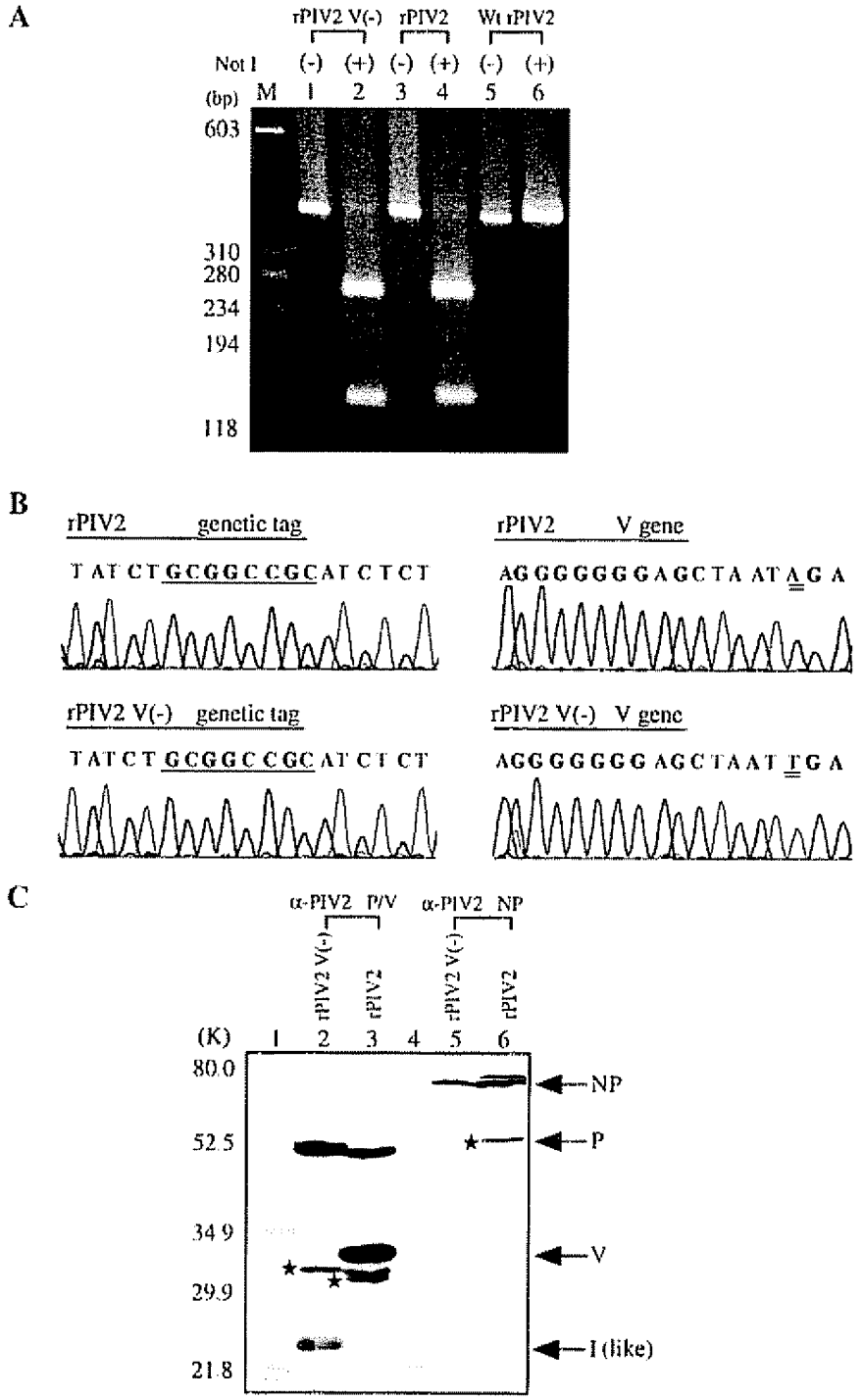

FIG 2 Identification of virus rescue by RT-PCR amplification and by Western blotting from rPIV2- or rPIV2V(−)-infected Vero cells (A) Demonstration of the genetic tag in the genome of transfectant recombinant viruses. rPIV2 and rPIV2V(−): template RNAs for the amplifications were obtained from rPIV2V(−) (lanes 1 and 2), from rPIV2 (lanes 3 and 4), or from wtPIV2 (lanes 5 and 6) The amplified DNA spanning the *NotI* site on the virus genome was separated on a 2% agarose gel before (lanes 1, 3, and 5) and after digesting with *NotI* (lanes 2, 4, and 6) (B) Nucleotide sequencing of genetic tag and mutated site in the V gene from RT-PCR products: RT-PCR products of recombinant viruses shown in (A) were subcloned and sequenced on an ABI 310 sequencer according to the manufacturer's protocols Top row shows the nucleotide sequences of the region around genetic tag and around RNA-editing site of rPIV2 genome RNA. Bottom row shows the nucleotide sequences over the same regions of rPIV2V(−) genome RNA (C) Absence of V protein synthesis in Vero cells infected with the rPIV2V(−): the lysates of Vero cells infected with the rPIV2V(−) (lanes 2 and 5) or rPIV2 (lanes 3 and 6) were analyzed by Western blotting with hPIV2-specific monoclonal antibodies against P/V (lanes 2 and 3) and NP proteins (lanes 5 and 6) Lane 1: molecular markers * indicate the degraded products of the P or NP protein

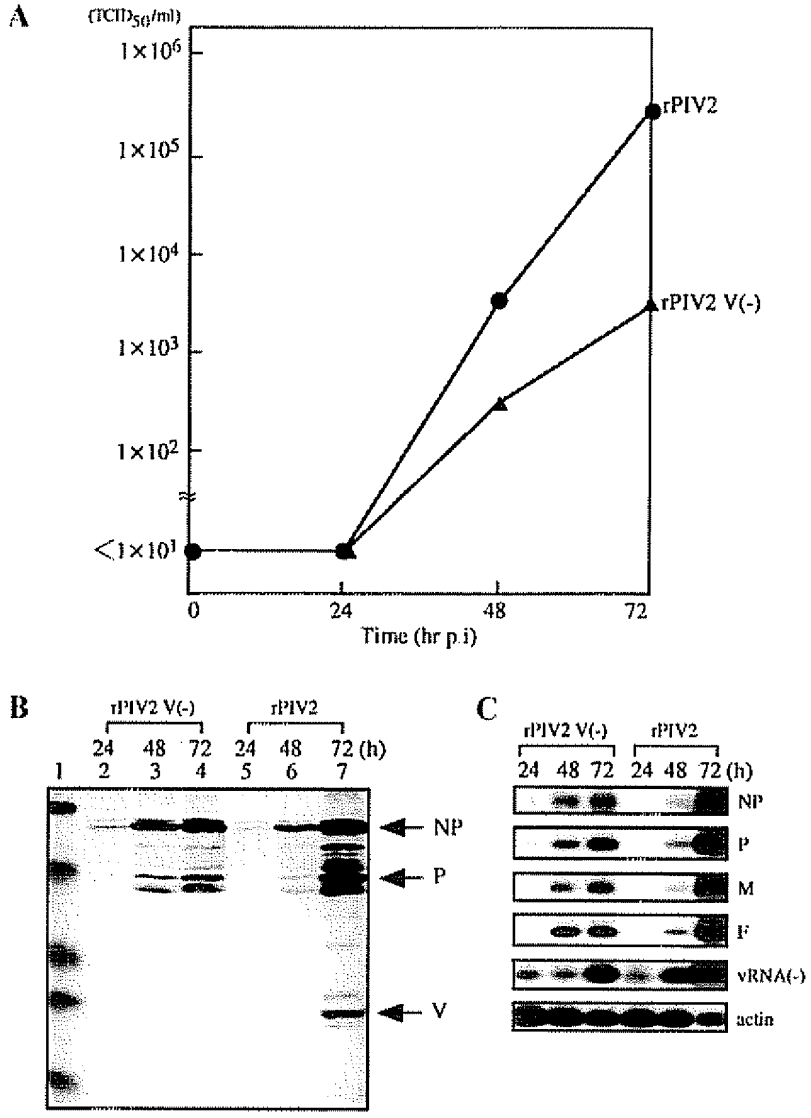

FIG 3 Replication of rPIV2 or rPIV2V(−) in Vero cells under multiple cycle growth conditions (m o i of 0 001) (A) The virus growth of rPIV2 and rPIV2V(−) in Vero cells: after infection with an m o i of 0 001, virus titers were determined at the indicated time points by CPE method using Vero cells and virus titers were expressed as 50% tissue culture infectious dose ($TCID_{50}$) The titer of rPIV2 is indicated by solid circles (●) and that of rPIV2V(−) by solid triangles (▲) (B) Western blot analysis: expressions of the NP, P, and V proteins were analyzed in rPIV2V(−)-infected (lanes 2–4) and rPIV2-infected Vero cells (lanes 5–7) by Western blot analysis using anti-hPIV2 P/V and anti-hPIV2 NP antibodies Lane 1: molecular markers (C) The kinetics by RT–PCR/Southern of viral mRNAs and genomic RNA derived from rPIV2V(−) or rPIV2 using hPIV2- and actin-specific probes Expressions of the viral mRNAs and genomic RNA were analyzed in Vero cells infected with rPIV2V(−) or rPIV2 for 24, 48, and 72 h

and rPIV2 were 175 and 165 nm, respectively, which shows the typical character of paramyxovirus virions Typical nucleocapsid strands were also clearly seen, and they were enclosed with the envelope as typical paramyxoviruses On the other hand, the rPIV2V(−) particles consisted of greatly heterogeneous population, and mean diameter of rPIV2V(−) was 222 nm Furthermore, larger spherical particles with a diameter of ~250 ~ 500 nm were frequently found (Figs 4C and 4D) Diameter was significantly larger in rPIV2V(−) particles than in either wtPIV2 or rPIV2 particles ($P < 0\ 05$).

Growth of rPIV2 and rPIV2V(−) in CV-1 and FL cells

It has recently been reported that the V protein of SV5 inhibits interferon (IFN) signaling by targeting STAT1 (Didcock *et al.*, 1999) Thus, we investigated whether the growth of rPIV2V(−), which is defective in the unique V-specific cysteine-rich C terminus, was affected by IFN system Under multiple-step replication conditions with an m o i of 0 01, the growth of rPIV2V(−) in CV-1 and FL cells was compared with that of rPIV2. rPIV2 could replicate efficiently in CV-1 and FL cells, although maximum titers of rPIV2 in FL cells were reduced by ~10-fold as much as those in CV-1 cells (Figs 5A and 5B) On the

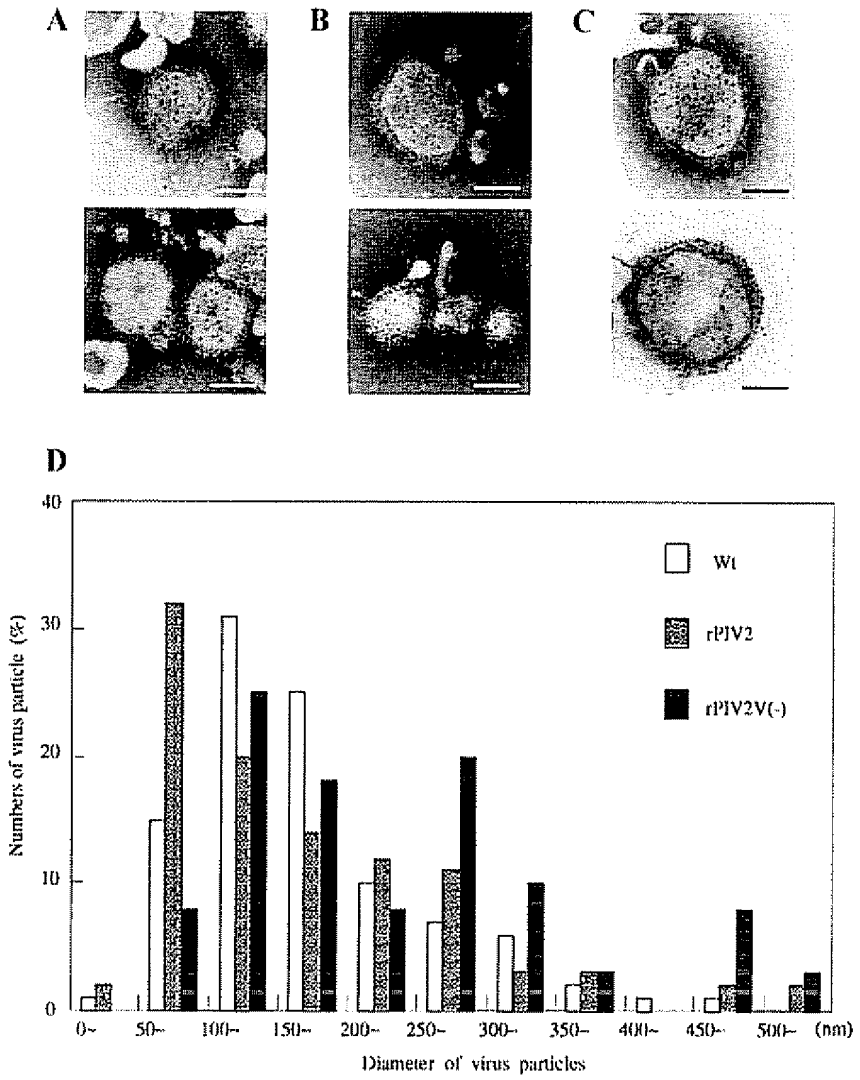

FIG 4. Electron microscopy of viral particles (A–C) The purified virions of wtPIV2 (A), prPIV2 (B), and rPIV2V(−) (C) were observed by an electron microscopy using anti-hPIV2 HN monoclonal antibody. Bars indicate 100 nm (D) The diameters of wtPIV2, rPIV2, and rPIV2V(−) virions were measured

contrary, no growth of rPIV2V(−) could be detected in both cells Since Vero cells were regarded as an IFN nonresponder, we investigated whether the neutralization of the endogenously produced IFN might affect the yield of rPIV2V(−) in CV-1 and FL cells As shown in Figs 5A and 5B, anti-IFN-β antibody shows no effect on the growth of rPIV2 in CV-1 and FL cells On the other hand, the yield of rPIV2V(−) was clearly enhanced in the presence of anti-IFN-β antibody (Figs 5A and 5B), although maximum titer of rPIV2V(−) was lower than that of rPIV2 These observations indicate that the rPIV2V(−) is highly sensitive to IFN and that no growth of rPIV2V(−) in CV-1 and FL cells is mainly due to its hypersensitivity to endogenously produced IFN

When FL cells was infected with rPIV2V(−), CPE developed during first 2 days and thereafter cell damage was cured (Fig 6A), indicating that the growth of the V knockout hPIV2 in FL cells is self limited However, the cell damage induced by rPIV2V(−) was expanded in Vero cells as the infected cells were cultured and finally the greater part of the cells died (Fig 6A) On the contrary, infection of Vero and FL cells with rPIV2 resulted in the total cell death (Fig 6B) Interestingly, anti-IFN-β antibody partially enhanced the rPIV2V(−)-induced CPE in FL cells These findings show that self-limiting growth of V(−) hPIV2 is, in part, related to IFN

DISCUSSION

In this work, we established the recovery system of infectious recombinant hPIV2 (rPIV2) from the full-length cDNA constructs In addition, we isolated the V protein knockout hPIV2 [rPIV2V(−)], which is defective in the V-specific cysteine-rich domain by introducing mutations (stop codon) into the full-length cDNA genome

The genome length of members belonging to the genera *Respirovirus* and Rubulavirus is known 6n length, called the "rule of six" (Calain and Roux, 1993) However,

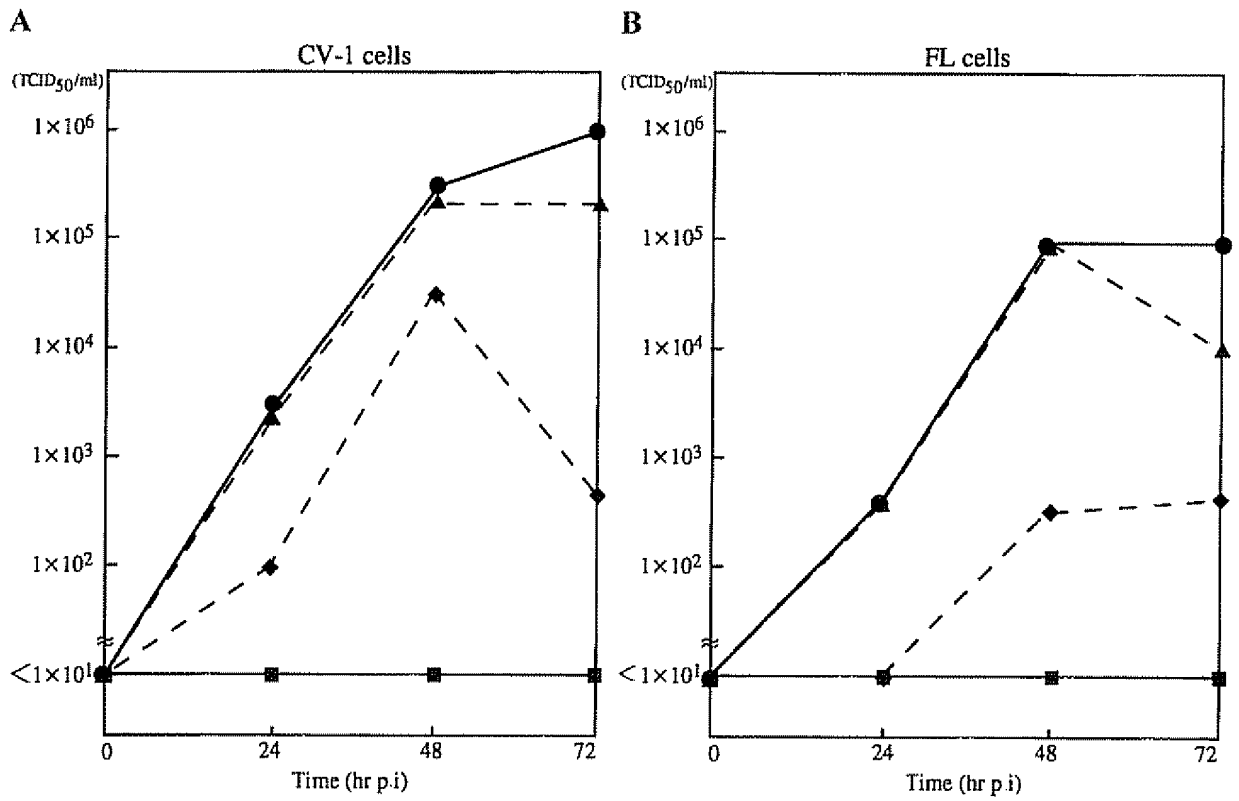

FIG 5 Growth of rPIV2 and rPIV2V(−) in CV-1 and FL cells CV-1 (A) and FL cells (B) were infected with rPIV2 (●, ▲) or rPIV2V(−) (■, ◆) under multiple-cycle growth conditions (an m o i of 0 01) in the presence (- - -) or absence (—) of anti-IFN-β antibody The virus titers were determined at the indicated time points by CPE method using Vero cells and virus titers were expressed as 50% tissue culture infectious dose ($TCID_{50}$)

the previously reported hPIV2 genome length were not 6n length Hence, at least three different RT–PCR clones of hPIV2 were sequenced, and one nucleotide deletion was found in 3′-noncoding region of HN gene in previously reported hPIV2 sequence (data not shown) This result indicated the total number of nucleotides in the correct hPIV2 genome was a multiple of six For SV, it was found that efficient replication of the genome only occurred when the total nucleotide numbers obey the "rule of six" (Calain and Roux, 1993) However, Murphy and Parks (1997) suggested that the genome of 6n length was a preference but not absolute requirement for SV5 replication Although the full-length cDNA used for the rescue of rPIV2 was not 6n length (15,665 nt including genetic tag), infectious virus could be isolated, and the recombinant virus could efficiently replicate in various cells The full-length cDNA has one nucleotide deletion in the 3′-noncoding region of M gene Nevertheless, the replication and transcription of rPIV2 showed the kinetics comparable to wtPIV2 (unpublished data) Thus, likewise SV5, hPIV2 does not appear to absolutely obey to the "rule of six," suggesting that 6n length genome has less significance in *Rubulavirus* than in *Respirovirus*

For the rescue of hPIV2 from cDNA, we used the strategy performed by Schnell *et al* (1994), who transfected the anti-genome RNA strand of rabies virus to the cells The rescues of infectious recombinant virus from cDNAs of SV and hPIV3 in the genus Respirovirus, SV5, MuV, and NDV in the genus *Rubulavirus* have recently succeeded by application of their system To establish the recovery system of hPIV2 from cDNA clone, we employed the methods that were used for the isolation of recombinant SV5 closely related to hPIV2 (He *et al.*, 1997) Since the efficiency of the rescue of rPIV2 was poor as compared with other recombinant viruses, we were not able to isolate infectious virus in the supernatant of first cells (~5 × 10^6) transfected with plasmids complex Accordingly, we attempted to coculture whole transfected cells with fresh Vero cells (the ratio of 1:1) for 2 days After this cocultivation, we could isolate a few infectious viruses in the culture fluids

The P gene is the second proximal to the 3′ terminus in virus genome in the family *Paramixoviridae* and frequently encodes one or more other small polypeptides, and also RNA-editing to insert pseudotemplated G residues is found for all the three paramyxovirus genera The C protein translated from an overlapping open reading frame of P protein exists in two respirovirus, SV and hPIV3 (Giorgi *et al*, 1983; Spriggs and Collins, 1986) In *Respirovirus*, other than hPIV1 (Matsuoka *et al*, 1991), the P mRNA is correct transcript from the vRNA, and the V mRNA is edited mRNA In rubulavirus including hPIV2, however, the C ORF is missing, the V protein is encoded by the faithful copy from the vRNA, and the P protein is

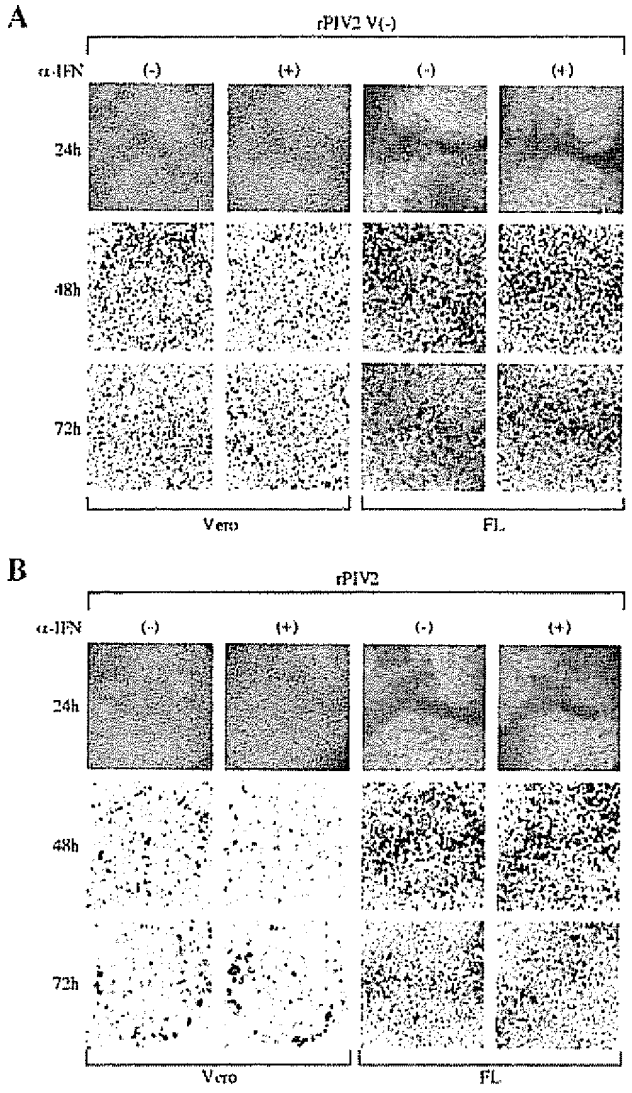

FIG 6 Cytopathogenic effect induced by rPIV2 and rPIV2V(−) CV-1 and FL cells were infected with rPIV2V(−) (A) or rPIV2 (B) under multiple-cycle growth conditions (an m o i of 0 01) in the presence or absence of anti-IFN-β antibody After incubation for 24, 48, and 72 h, the cells were fixed and stained with Giemsa's solution

encoded by the mRNA after pseudotemplated transcriptional addition of nucleotides Furthermore, the hPIV2 V protein is a structural protein detected in the virion (Ohgimoto *et al.*, 1990), whereas the V and C proteins of SV were nonstructural proteins (Lamb and Choppin, 1977) Kato *et al* (1997) reported that V(−) SV showed remarkably attenuated *in vivo* replication capacity and pathogenicity for mice and proposed that the V protein has a luxury function required for *in vivo* replication In addition, Gotoh *et al* (1999) have recently reported that the 4C knockout SV completely loses the ability to suppress the IFN signaling However, the cells infected with V(−) SV showed a high resistance to IFN activity (Gotoh *et al*, 1999) These findings show that the C protein plays an important role in blocking IFN-mediated cellular responses

On the contrary, Didcock *et al* (1999) have reported that expression of the SV5 V protein, a structural protein, inhibits IFN signaling and induces the degradation of STAT1 in the absence of other virus proteins However, since the expression of only SV5 P protein sharing the first 164 amino acid with V protein did not block the IFN signaling (Didcock *et al*, 1999), we speculated that the V-protein-specific cysteine-rich region participates in blocking the IFN signaling Therefore, we created rPIV2V(−) devoid of the V-specific cysteine-rich domain and analyzed the properties of rPIV2V(−) The rPIV2 could efficiently replicate in Vero, CV-1, and FL cells, while rPIV2V(−) did not multiply in CV-1 and FL cells at all The rPIV2V(−) is able to grow in only Vero cells known as IFN nonresponders In rPIV2V(−)-infected FL cells, the growth of rPIV2 was clearly recovered, though of lower yields, by adding anti-IFN-β antibody to the supernatant of those cells These findings indicate that no replication of rPIV2V(−) in CV-1 and FL cells is mainly due to interference by the endogenously produced IFN during the course of infection, and the resistance against IFN can not be induced by infection with rPIV2V(−) This shows that the V-specific cysteine-rich C terminus of the hPIV2 V protein, composed of 58 amino acids, possesses the function blocking the antiviral action by IFN

Kurotani *et al* (1998) have reported that the SV C proteins are categorically nonessential gene products but greatly contribute to full replication in tissue culture cells and pathogenicity for mice Furthermore, Hasan *et al* (2000) have reported that the accessory and basically nonstructural C proteins are critically required in the SV assembly process, being out of all relation to function to counteract the antiviral action of interferon-α/β Since Vero cells are known as an IFN nonproducer, we investigated whether relatively low virus yield of rPIV2V(−) in Vero cells was related to the inefficient assembly of the rPIV2V(−) Under the multiple-cycle growth conditions, rPIV2V(−) slowly replicated in Vero cells, and the virus yield was reduced by ~1 log (48 h p i) and 2 logs (72 h p i) lower than those of the rPIV2 On the other hand, the syntheses of viral mRNAs and proteins were larger in quantity in rPIV2V(−) infection than that in rPIV2 infection until 48 p i , while at 72 h p i , the situation was reversed Furthermore, there were no differences between the vRNA syntheses of rPIV2V(−) at 24 and at 48 h p i , while amounts of other viral components increased together from 24 to 48 h p i These findings indicate that the infectivity of the progeny virus of rPIV2V(−) decreases but genomic RNA synthesis ability of rPIV2V(−) is not impaired Also, though the infective titer of rPIV2V(−) at 72 h p i was almost the same with that of rPIV2 at 48 h p i , the synthetic quantities of the viral proteins and mRNAs of rPIV2V(−) at 72 h p i were clearly detected with much larger than those of rPIV2 at 48 h p i These suggest that the assembly process is impaired in rPIV2V(−)-infected cells This speculation was sup-

ported by observation of viral morphology using an immunoelectron microscopy. The rPIV2 and wtPIV2 showed the typical character of paramyxovirus, possessing a relatively homogenous population of spherical particles with a diameter of ~100–200 nm, whereas rPIV2V(−) displayed the heterogeneous population, and large spherical particles (250–500 nm) were frequently found. Thus, it is inferred that the V-specific cysteine-rich domain of C terminus of V protein plays an important role in the assembly, maturation, and morphogenesis of hPIV2.

As described above, the hPIV2 V protein will be required in the assembly and maturation process. In paramyxoviruses, the M protein has been thought to play a critical role in assembly and maturation. The C protein of SV responsible for virus assembly colocalized with the M and HN proteins perfectly and pretty well, respectively (Hasan *et al*., 2000). Furthermore, the internal proteins, the P and L proteins, were found to distribute diffusely throughout the cytoplasm and HN tended to aggregate on the cell surface in 4C(−) SV-infected CV-1 cells (Hasan *et al*., 2000). On the contrary, the V protein of hPIV2 was detected dominantly in the nucleus (Watanabe *et al*., 1996) and has an ability of associating with the internal protein, NP protein (Nishio *et al*., 1996). When Vero cells were infected with rPIV2V(−) and stained with anti-NP or anti-P antibody, both viral proteins were found to distribute granularly throughout the cytoplasm similar to those found in rPIV2 and wtPIV2 (data not shown). Subsequently, the HN protein was stained on the rPIV2- and rPIV2V(−)-infected cells. The staining pattern of rPIV2V(−) HN protein was different from that of rPIV2 HN protein; that is, the specific fluorescence was glanular in rPIV2-infected cells, while it showed diffuse pattern in rPIV2V(−)-infected cells (unpublished data). However, the distribution pattern of the M protein could not be identified due to low titer of our antibody. It remains to be defined whether the association of the V protein with the HN or M protein is required for the assembly processes.

Several properties have been ascribed to the V protein of *Paramyxovirus*. The V protein of hPIV2 has one binding domain to the NP protein in the P–V common domain, which is located in the N-terminal region, aa1–46 (Nishio *et al*., 1996). The V protein in the cells infected with hPIV2 or transfected with the V-specific cDNA clone localized in the nuclei of the cells (Watanabe *et al*., 1996; Nishio *et al*., 1997, 1999). Two noncontiguous regions in the hPIV2 V protein, aa1–46 and aa175–196 (cysteine-rich V-specific domain), are required for nuclear localization and retention (Watanabe *et al*., 1996). The V protein of SV5 also interacts with both viral NP and cellular proteins (damage-specific DNA binding protein) (Lin *et al*., 1998). Recently Lin and Lamb (2000) have reported that slow progression of the cell cycle is observed in the cells infected with SV5 or expressing the SV5 V protein. In addition, the V protein of SV5 has been recently reported to be responsible for the virus-mediated inhibition of IFN signaling (Didcock *et al*., 1999). We clarified in this study that the V protein of hPIV2 also has an ability of counteracting IFN action. Young *et al*. (2000) have recently reported that there is a specific loss of STAT2 in hPIV-2-infected cells. Furthermore, the V protein of hPIV2 was found to be related to virus assembly and morphogenesis.

In summary, we established the reverse genetic technique of hPIV2 and isolated infectious recombinant viruses from the cDNA clones. Furthermore, we isolated and analyzed the V protein knockout hPIV2. Replication of rPIV2V(−) was self-limiting in the culture cells. At least two mechanisms play important roles in self-limiting growth of rPIV2V(−), namely, (1) the rPIV2V(−) is highly sensitive to IFN and that no growth of rPIV2V(−) in CV-1 and FL cells is mainly due to its hypersensitivity to endogenously produced IFN and (2) virus assembly is impaired in rPIV2V(−)-infected cells.

MATERIALS AND METHODS

Cells and virus preparations

Monolayer cultures of Vero, CV-1, and FL cells and human parainfluenza type 2 virus (hPIV2), Toshiba strain, were used in this study. Virus propagation and purification were performed as previously reported (Ito *et al*., 1987). Chick embryo fibroblasts (CEF) were grown as described previously (Ito *et al*., 1974). Modified vaccinia virus Ankara (MVA) expressing bacteriophage T7 RNA polymerase, which was kindly provided by Dr. Bernard Moss (National Institutes of Health, Bethesda, MD) was grown in CEF cells (Sutter *et al*., 1995; Wyatt *et al*., 1995).

Reverse transcriptase–PCR amplification (RT–PCR)

Total RNA was extracted from hPIV2-infected Vero cells using TRIZOL Reagent (Gibco-BRL), according to the manufacturer's protocol. Reverse transcription (RT) was performed with SuperScriptII (Gibco-BRL). The oligonucleotide primers that annealed to the vRNA(−) strand were described in Table 1. Before reverse transcription, 5 μg of RNA and 20 pmol of primer were incubated at 70°C for 5 min and then chilled on ice. RT reaction mixtures contained this heat-denatured RNA and 200 U reverse transcriptase in a final volume of 20 μl were incubated at 42°C for 45 min. Five to 10 μl of RT product was then amplified in PCR reaction (a total reaction volume of 50 μl) using appropriate oligonucleotide primer pairs (see Table 1 and Fig. 1). Briefly, the RT product and the primers were preheated at 94°C for 1 min and then subjected to 30 cycles, each cycle at 98°C for 30 s and 68°C for 15 min using Takara LA *Taq* DNA polymerase.

Construction of a full-length cDNA of the hPIV2 genome

To facilitate the ligation of long RT–PCR products, we improved the multi-cloning site (MCS) in pUC118 plasmid vector. Namely, the MCS was replaced with the following double strand oligonucleotides. 5′-GAATTCGATATCGTCGACGCATGCGAGCTCAGATCTGCG GCCGCGGTACCCTCGAGGGTTACCGGATCCCCCGGGAAGCTTGGG-3′, annealed to its complementary oligonucleotide. The resulting restriction enzyme site order is 5′-*Eco*RI-*Eco*RV-*Sal*I-*Sph*I-*Sac*I-*Bgl*II-*Not*I-*Kpn*I-*Xho*I-*Bst*PI-*Bam*HI-*Sma*I-*Hin*dIII-3′. We named this improved vector pUCRVG. We synthesized appropriate oligonucleotide primer pairs as shown in Table 1 and performed the RT–PCR using vRNA as a template. As shown in Fig. 1, cDNA fragment 1 of five RT–PCR products overlapping at the restriction enzyme site was amplified by PCR using the primer 1 (positive sense), which included 5′-*Sal*I site-T7 promoter (17nt)-3G-hPIV2 leader sequence (21nt), while the primer 2 (negative sense) contained 5′-*Not*I site (extra nucleotides for genetic tag)-AGAT (extra nucleotides for rule of six)-complementary to hPIV2 nt 102–125. Likewise, fragment 2 was amplified using the primer 3 including 5′-*Not*I-hPIV2 nt 126–151 (on NP gene) and the primer 4 including complementary to hPIV2 nt 2659–2691 (containing *Sac*I on V/P gene). Fragments 3 and 4 were amplified using the primer pairs 5 (nt 2659–2691) and 6 (complementary to nt 8122–8154) and 7 (nt 8122–8154) and 8 (complementary to nt 10,012–10,045) containing the restriction enzyme sites described above, *Sac*I, *Kpn*I, *Kpn*I and *Bst*PI, respectively. Fragment 5 was amplified using the primer pairs 9 (nt 10,012–10,045) and 10, which included the sequence complementary to 5′-*Sma*I site-hepatitis delta virus ribozyme (84 nt)-hPIV2 trailer (27 nt). These RT–PCR fragments were digested with restriction enzymes in both termini and subcloned into pUC118RVG plasmid vector. Then the plasmid including the full-length hPIV2 cDNA genome (pPIV2) was constructed by stepwise assembly from these subcloned plasmid DNAs using standard molecular biology techniques.

Construction of hPIV2 genome cDNA devoid of V gene

To generate a stop codon in 8 nt downstream of the RNA-editing site of the V gene, one nucleotide change (AGA to TGA) was introduced into the plasmid pPIV2 containing the cDNA copy of the full-length hPIV2 antigenome. Consequently, the following primers, a pair of complementary oligonucleotide primers, (5′-GGGAGCTAATtG AGAAAGAGCAAG-3′ and 5′-CTTGCTCTTTCTCaATTAGCTCCC-3′), which contained a mutation (shown by lower-case letters) and outer primers, 5′-GCTGTTCTAACTAGTGCAG CGGAT-3′ (including *Spe*I site) and 5′-CAAGTCCCTTTAAGAGCTCAATGATCTCCTTC A-3′ (including *Sac*I site) were used for generation of the mutated DNA fragment by two-step PCR-based overlap primer extension (Steffan *et al.*, 1989). PCR was performed with the template, pPIV2, for 25 cycles of 94°C, 1 min, 60°C, 1 min, and 72°C, 1 min. The amplified fragments were digested with *Spe*I and *Sac*I and inserted into similarly digested plasmid including the RT–PCR fragment 2 described above (Fig. 1) and then similarly inserted into pPIV2 digested with *Not*I and *Sac*I. This plasmid was designated pPIV2V(−).

Transfection and recovery of viruses from cDNAs

Vero cells at 90–95% confluence in a six-wells plate were infected with MVA at an m.o.i. of 1, and after 2 h, the plasmid pPIV2 and the plasmids encoding NP, P, and L, which were under control of T7 promoters in bluescriptII vector (p2NP, p2P, and p2L, respectively) were transfected into cells with LIPOFECTAMINE 2000 Reagent (Gibco–BRL). The amounts of plasmids were as follows: 1 μg pPIV2, 2 μg p2NP, 0.25 μg p2P, and 2 μg p2L per well. After incubation for 6 h, the transfection media were changed to MEM containing 5% fetal calf serum. After incubation for 40 h at 37°C, the cells were detached and harvested by a cell scraper and then were cocultured with fresh Vero cells. After 2 days of incubation, the media were harvested and then further inoculated into Vero cells, and the supernatant was filtered through 0.22 μM filter to remove MVA. High-titer virus was generated by infecting Vero cells or CV-1 cells, which was used as the stock virus for all the experiments. The hPIV2 recovered from pPIV2 cDNA was designated rPIV2. Also, the hPIV2 recovered from pPIV2V(−) cDNA by using the same manipulation was designated rPIV2V(−).

RT–PCR/Southern blot analysis

Total RNA was extracted with Trisol reagent (Gibco–BRL). RNA yields were determined spectrophotometrically. The RNA from rPIV2- or rPIV2V(−)-infected cells was examined using RT–PCR followed by Southern blotting with an hPIV2-specific radioactive probe. Five-microgram aliquots of each RNA preparation were reverse transcribed by the SuperscriptII reverse transcriptase (Gibco–BRL) and oligo dT- or primer 1 (Table 1) in a reaction volume of 20 μl. Five microliters of RT products were used as PCR templates. The rPIV2 or rPIV2V(−)-specific fragment was amplified with the appropriate primer. Thermal cycling conditions were as follows: the reaction mixture was incubated at 94°C for 1 min followed by 15 cycles of 94°C for 1min, 60°C 1 min, and 72°C for 1.5 min using Takara EX *Taq* DNA polymerase in a reaction volume of 100 μl.

After PCR amplification, 10 μl of the total reaction were fractionated on a 1% agarose gel and transferred onto a Hybond N^+ membrane (Amersham) under alkaline conditions. The RT–PCR products were visualized by South-

ern hybridization with the hPIV2- and actin-specific probes

Western blot assays and antibodies

The virus-infected cells were washed twice with PBS, dissolved with 100 μl of sample buffer [100 mM Tris–HCl (pH 6 8), 4% SDS, 20% glycerol, 0 1% bromphenol blue, 10% 2-mercaptoethanol] per well in 12-well plate and sonicated Twenty-five microliters of the 100 μl cell lysates were analyzed by SDS–PAGE and electroblotted onto nitrocellulose membranes The membranes were blocked with 5% skim milk in PBS, treated with each monoclonal antibody (MAb) at room temperature for 1 h, washed three times with 0.05% Tween 20 in PBS (PBS/T), and treated with biotinylated secondary antibody for 30 min After washing with PBS/T, the membranes were treated with avidin-biotin-peroxidase complex (Vector Laboratories) at room temperature for 30 min After washing with PBS, the membranes were immersed in PBS containing 0 3% 4-chloro-1-naphthol and 0 009% hydrogen peroxide For Western blot assays, MAbs against hPIV2-NP (64-1A) and -P/V (315-1) (Tsurudome *et al.*, 1989) were used

Virus purification

When the virus-infected Vero cells showed extensive CPE, a large amount of culture supernatant (500 ml) of the cells was collected and then was centrifuged at 1500 *g* for 5 min at 4°C The surpernatant was centrifuged again at 10.000 *g* for 12 h at 4°C The virus pellet resuspended in TNE buffer [50 mM Tris–HCl (pH 7.5). 150 mM NaCl, 1 mM EDTA] was layered on a 50%/20% (w/w) discontinuous sucrose density gradient in TNE buffer and centrifuged at 100,000 *g* for 12 h at 4°C Sucrose fraction (1 ml) was collected from the interface between 50 and 20% sucrose. The sucrose fraction was diluted in 12 ml of PBS (pH 7 4). and spun down at 100,000 *g* for 2 h at 4°C The pellet was suspended in 100 μl of PBS

Electron microscopy (EM) and immune electron microscopy (IEM)

For conventional EM, 3 μl of each virus sample was applied to Formvar-coated and carbon-vaporized grids and then negatively stained with 2% phosphotungstic acid (pH 6 5) The grid was assessed using a Hitachi H-800 electron microscope IEM was performed using mouse anti-hPIV2 HN monoclonal antibody as a primary antibody, and goat anti-mouse IgG colloidal gold particles (5-nm diameter; BioCell Research Laboratories, Cardiff, UK) as a secondary antibody Briefly, 3 μl of each virus sample was adsorbed on the grid and then the semidried grid was floated for 5 min on a drop of TBS–BSA [100 mM Tris–HCl (pH 7 6), 150 mM NaCl, and 2% bovine serum albumin] placed on a parafilm in a moist chamber The grid was then floated for 30 min on a drop of TBS containing 3% gelatin and then the excess of gelatin was washed away from the drop of TBS–BSA The grid was incubated for 60 min on a drop of primary antibody solution (diluted 1:100 in TBS–BSA) at room temperature and then washed three times with TBS–BSA After incubating the grid for 60 min in a drop of secondary antibody solution (diluted 1:20 in TBS–BSA), the grid was washed three times with TBS–BSA and once with TBS. The grid was negatively stained with 2% phosphotungstic acid for observation by an electron microscope

Viral replication in the presence of anti-IFN-β antibody

FL and CV-1 cells grown in 12-well plated were infected with rPIV2 or rPIV2V(−) at an m o i of 0 01. After 2 h of incubation, the inoculum was removed, and the cells were further cultured with MEM (supplemented with 1% FCS) containing antibody against human IFN-β (800 U/ml) or no antibody At 24, 48, and 72 h p.i , the titers of the virus were determined by CPE method using Vero cells, and virus titers were expressed as 50% tissue culture infectious dose ($TCID_{50}$).

REFERENCES

Calain, P., and Roux, L (1993) The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *J Virol* 67, 4822–4830

Cattaneo, R., Kaelin, K., Baczko, K., and Billeter, M A (1989) Measles virus editing provides an additional cysteine-rich protein *Cell* 56, 759–764

Clarke, D K, Sidhu, M S., Jhonson, J E., and Udem, S A (2000) Rescue of mumps virus from cDNA. *J Virol* 74, 4831–4838

Collins, P L., Hill, M G., Camargo, E., Grosfeld, H., Chanok, R M., and Murphy, B R (1995) Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development *Proc Natl Acad Sci USA* 92, 11563–11567

Didcock, L., Young, D F., Goodbourn, S., and Randall, R E (1999) The V protein of simian virus 5 inhibits interferon signaling by targeting STAT1 for proteasome-mediated degradation *J Virol* 73, 9928–9933

Durbin, A P., Hall, S L., Siew, J W., Whitehead, S S., Collins, P L., and Murphy, B R (1997) Recovery of infectious human parainfluenza virus type 3 from cDNA *Virology* 235, 323–332

Fuerst, T R., Niles, E G., Studier, F W., and Moss, B (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase *Proc Natl Acad Sci USA* 83, 8122–8126

Garcin, D., Ito, M., and Kolakofsky, D (1997) A point mutation in the Sendai virus accessory C proteins attenuates virulence for mice, but not virus growth in cell culture *Virology* 238, 424–431

Garcin, D., Pelet, T., Calain, P., Roux, L., Curran, J., and Kolakofsky, D (1995) A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: Generation of a novel copy-back nondefective interfering virus *EMBO J* 14, 6087–6094

Giorgi, C., Blumberg, B M., and Kolakofsky, D (1983) Sendai virus contains overlapping genes expressed from a single mRNA *Cell* 35, 829–836

Gotoh, B., Takeuchi, K., Komatsu, T., Yokoo, J., Kimura, Y., Kurotani, A., Kato, A., and Nagai, Y (1999) Knockout of the Sendai virus C gene

eliminates the viral ability to prevent the interferon-α/β-mediated responses *FEBS Lett.* 459, 205–210

Hasan, M. K., Kato, A., Muranaka, M., Yamaguchi, R., Sakai, Y., Hatano, I., Tashiro, M., and Nagai, Y. (2000) Versatility of the accessory C proteins of Sendai virus: Contribution to virus assembly as an additional role *J. Virol.* 74, 5619–5628

He, B., Paterson, R. G., Ward, C. D., and Lamb, R. A. (1997) Recovery of infectious SV5 from cloned DNA and expression of a foreign gene *Virology* 237, 249–260

Hoffman, M. A., and Banerjee, A. K. (1997) An infectious clone of human parainfluenza virus type 3 *J. Virol.* 71, 4272–4277

Ito, Y., Kimura, Y., Nagata, I., and Kunii, A. (1974) Effects of L-glutamine deprivation on growth of HVJ (Sendai virus) in BHK cells *J. Virol.* 13, 557–566

Ito, Y., Tsurudome, M., Hishiyama, M., and Yamada, A. (1987) Immunological interrelationships among human and non-human paramyxoviruses revealed by immunoprecipitation *J. Gen. Virol.* 68, 1289–1297

Kato, A., Kiyotani, K., Sakai, Y., Yoshida, T., and Nagai, Y. (1997) The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis *EMBO J.* 16, 578–587

Kato, A., Sakai, Y., Shioda, T., Kondo, T., Nakanishi, M., and Nagai, Y. (1996) Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense *Genes Cells* 1, 569–579

Kawano, M., Bando, H., Ohgimoto, S., Kondo, K., Tsurudome, M., Nishio, M., and Ito, Y. (1990a) Sequence of the fusion protein gene of human parainfluenza type 2 virus and its 3′ intergenic region: Lack of small hydrophobic (SH) gene *Virology* 178, 289–292

Kawano, M., Bando, H., Ohgimoto, S., Okamoto, K., Kondo, K., Tsurudome, M., Nishio, M., and Ito, Y. (1990b) Complete nucleotide sequence of matrix gene of human parainhfluenza type 2 virus and expression of the M protein in bacteria *Virology* 179, 857–861

Kawano, M., Bando, H., Yuasa, T., Kondo, K., Tsurudome, M., Komada, H., Nishio, M., and Ito, Y. (1990c) Sequence determination of the hemagglutinin-neuraminidase (HN) gene of human parainfluenza type 2 virus and construction of a phylogenetic tree for HN proteins of all the paramyxoviruses that are infectious to humans *Virology* 174, 308–313

Kawano, M., Okamoto, K., Bando, H., Kondo, K., Tsurudome, M., Komada, H., Nishio, M., and Ito, Y. (1991) Characterizations of the human parainfluenza type2 virus gene encoding the L protein and the intergenic sequenses *Nucleic Acids Res.* 19, 2739–2746

Kawano, M., Tsurudome, M., Oki, N., Nishio, M., Komada, H., Matsumura, H., Kusagawa, S., Ohta, H., and Ito, Y. (1993) Sequence determination of the P gene of simian virus 41: Presence of irregular deletions near the RNA-editing sites of paramyxoviruses *J. Gen. Virol.* 74, 911–916

Kondo, K., Bando, H., Tsurudome, M., Kawano, M., Nishio, M., and Ito, Y. (1990) Sequence analysis of the phosphoprotein (P) genes of human parainfluenza type 4A and 4B viruses and RNA editing at transcript of the P genes: The number of G residues added is imprecise *Virology* 178, 321–326

Kretzschmar, E., Peluso, R., Schnell, M. J., Whitt, M. A., and Rose, J. K. (1996) Normal replication of vesicular stomatitis virus without C proteins *Virology* 216, 309–316

Kurotani, A., Kiyotani, K., Kato, A., Shioda, T., Sakai, Y., Mizumoto, K., Yoshida, T., and Nagai, Y. (1998) The paramyxovirus, Sendai virus, C proteins are categorically nonessential gene products but silencing their expression severely impairs viral replication and pathogenesis *Genes Cells* 2, 111–124

Lamb, R. A., and Choppin, P. W. (1977) The synthesis of Sendai virus polypeptides in infected cells II Intracellular distribution of polypeptides *Virology* 81, 371–381

Lawson, N., Stillman, E., Whitt, M., and Rose, J. (1995) Recombinant vesicular stomatitis viruses from DNA *Proc. Natl. Acad. Sci. USA* 92, 4471–4481

Lin, G. Y., Paterson, R. G., Richardson, C. D., and Lamb, R. A. (1998) The V protein of the paramyxovirus SV5 interacts with damage-specific DNA binding protein *Virology* 249, 189–200

Lin, G. Y., and Lamb, R. A. (2000) The paramyxovirus simian virus 5 V protein slows progression of the cell cycle *J. Virol.* 74, 9152–9166

Matsuoka, Y., Curran, J., Pelet, T., Kolakofsky, D., Ray, R., and Compans, R. W. (1991) The P gene of human parainfluenza virus type 1 encodes P and C proteins but not a cysteine-rich V protein *J. Virol.* 65, 3406–3410

Murphy, S. K., and Parks, G. D. (1997) Genome nucleotide lengths that are divisible by six are not essential but enhance replication of defective interfering RNAs of the paramyxovirus simian virus 5 *Virology* 232, 145–157

Nishio, M., Tsurudome, M., Ito, M., Kawano, M., Kusagawa, S., Komada, H., and Ito, Y. (1999) Isolation of monoclonal antibodies directed against the V protein of human parainfluenzavirus type 2 and localization of the V protein in virus-infected cells *Med. Microbiol. Immunol.* 188, 79–82

Nishio, M., Tsurudome, M., Ito, M., Watanabe, N., Kawano, M., Komada, H., and Ito, Y. (1997) Human parainfluenza virus type 2 phosphoprotein: Mapping of monoclonal antibody epitopes and location of the multimerization domain *J. Gen. Virol.* 78,1303–1308

Nishio, M., Tsurudome, M., Kawano, M., Ohgimoto, S., Ito, M., Komada, H., and Ito, Y. (1996) Interaction between nucleocapsid protein (NP) and phosphoprotein (P) of human parainfluenza virus type 2: One of the two NP binding sites on P is essential for granule formation *J. Gen. Virol.* 77, 2457–2463

Ohgimoto, S., Bando, H., Kawano, M., Okamoto, K., Kondo, K., Tsurudome, M., Nishio, M., and Ito, Y. (1990) Sequence analysis of P gene of human parainfluenza type 2 virus: P and cysteine-rich proteins are translated by two mRNAs that differ by two non-templated G residues *Virology* 177, 116–123

Paterson, R. G., and Lamb, R. A. (1990) RNA editing by G-nucleotide insertion in Mumps virus P-gene mRNA transcripts *J. Virol.* 64, 4137–4145

Pattnaik, A. K., Ball, L. A., LeGrone, A. W., and Wertz, G. W. (1992) Infectious defective interfering particles of VSV from transcripts of a cDNA clone *Cell* 69, 1011–1020

Peeters, B. P., de Leeuw, O. S., Koch, G., Gielkens, A. L. (1999) Rescue of Newcastle disease virus from cloned cDNA: Evidence that cleavability of the fusion protein is a major determinant for virulence *J. Virol.* 73, 5001–5009

Radecke, F., and Billeter, M. A. (1996) The nonstructural C protein is not essential for multiplication of Edmonston B strain measles virus in cultured cells *Virology* 217, 418–421

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G., and Billeter, M. A. (1995) Rescue of measles viruses from cloned DNA *EMBO J.* 14, 5773–5784

Schnell, M. J., Mebatsion, T., and Conzelmann, K.-K. (1994) Infectious rabies viruses from cloned cDNA *EMBO J.* 13, 4195–4203

Spriggs, M., and Collins, P. L. (1986) Sequence analysis of the P and C protein genes of human parainfluenza virus type 3: Patterns of amino acid sequence homology among paramyxovirus proteins *J. Gen. Virol.* 67, 2705–2719

Steffan, N. H., Henry, D. H., Robert, M. H., Jeffrey, K. P., and Larry, R. P. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction *Gene* 77, 51–59

Sutter, G., Ohlmann, M., and Erfle, V. (1995) Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase *FEBS Lett.* 371, 9–12

Thomas, S. M., Lamb, R. A., and Paterson, R. G. (1988) Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV 5 *Cell* 54, 891–902

Tsurudome, M., Nishio, M., Komada, H., Bando, H., and Ito, Y. (1989) Extensive antigenic diversity among human parainfluenza type 2 virus isolates and immunological relationships among paramyxoviruses revealed by monoclonal antibodies *Virology* 171, 38–48

Vidal, S., Curran, J., and Kolakofsky, D. (1990) Editing of the Sendai virus

P/C mRNA by G insertion occurs during mRNA synthesis via a virus-encoded activity. *J Virol* 64, 239–246

Watanabe, N., Kawano, M., Tsurudome, M., Kusagawa, S., Nishio, M., Komada, H., Shima, T., and Ito, Y. (1996) Identification of sequence responsible for nuclear targeting of the V protein of human parainfluenza virus type 2. *J Gen. Virol* 77, 327–338

Whelan, S. P., Ball, L. A., Barr, J. N., and Wertz, G. T. (1995) Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proc Natl Acad. Sci USA* 92, 8388–8392

Wyatt, L. S., Moss, B., and Rozenblatt, S. (1995) Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology* 210, 202–205

Young, D. F., Didcock, L., Goodbourn, S., and Randall, R. E. (2000) Paramyxoviridae use distinct virus-specific mechanisms to circumvent the interferon response. *Virology* 269, 383–390

Yuasa, T., Bando, H., Kawano, M., Tsurudome, M., Nishio, M., Kondo, K., Komada, H., and Ito, Y. (1990) Sequence analysis of the 3′ genome end and NP gene of human parainfluensa type 2 virus: Sequence variation of the gene-starting signal and the conserved 3′ end. *Virology* 179, 777–784